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A Role for *Xlim-1* in Pronephros Development in *Xenopus laevis*

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Xlim-1, **a LIM class homeobox gene expressed in *Xenopus laevis,* is one of the earliest known marker genes of pronephros development and is expressed in pronephros rudiment. In this study, we examined the role of *Xlim-1* in pronephros development. Temporal expression of *Xlim-1* in explants was analyzed in a series of induction assays using RT-PCR analysis. *Xlim-1* was expressed 9 to 15 h after activin/retinoic acid treatment, corresponding to pronephros differentiation in explants. We further examined the role of *Xlim-1* using a series of microinjection experiments. Presumptive pronephric anlagen of embryos were injected with various *Xlim-1* mutants, and effects of these *Xlim-1* mutants on pronephrogenesis in embryos and in explants were analyzed by RT-PCR and immunohistochemistry. Dominant-negative *Xlim-1* inhibited differentiation of pronephros in activin/retinoic acid-treated animal caps. In embryos injected with a dominant-negative form of *Xlim-1,* development of pronephric tubules was inhibited at the late tail-bud stage. Our results suggest that *Xlim-1* may not initiate differentiation of the pronephros, but that it is necessary for growth and elongation in the development of pronephric tubules.**

Key Words: Xenopus laevis; **pronephros; pronephric tubules; activin; retinoic acid; *Xlim-1*.**

INTRODUCTION

Homeobox genes regulate various steps in embryogenesis, including body axis determination, regional specification, and tissue or cell type specification. Homeobox genes have been divided into several subfamilies based on amino acid sequence similarities in the homeodomain, each of which may have distinct functions during development, and in the adult. Certain homeobox gene subfamilies are identified by conserved domains in addition to the homeodomain, as exemplified by the Pax (Gruss and Walther, 1992), POU (Schöler, 1991), and LIM class genes. The LIM class of homeobox genes, initially identified by sequence homology between the *Caenorhabditis elegans* genes *mec-3* and *lin-11* and the rat DNA binding factor *Isl-1*, is defined by the association of two tandemly repeated copies of a cysteine-rich domain with a homeodomain (Way and Chalfie, 1988; Freyd *et al.*, 1990; Karlsson *et al.*, 1990). The LIM family now includes the *Xenopus* genes *Xlim-1*, *Xlim-2*, and *Xlim-3* and the mouse homologue *lim-1* (Taira *et al.*, 1992; Fujii *et al.*, 1994), the *Drosophila* gene *apterous* (Cohen *et al.*, 1992; Bourgouin *et al.*, 1992), and the rodent genes *lmx-1* (German *et al.*, 1992) and *LH-2* (Xu *et al.*, 1993). The *C. elegans* LIM genes are required for the development of specific cell lineages, *mec-3* for mechanosensory neurons (Way and Chalfie, 1988) and *lin-11* for vulval cells (Ferguson *et al.*, 1987; Freyd *et al.*, 1990). The *apterous* gene functions in wing differentiation and development of a subset of embryonic muscles (Cohen *et al.*, 1992; Bourgouin *et al.*, 1992). Taken together, these observations suggest that LIM class homeobox genes in invertebrates mediate the specification and differentiation of specialized cell types.

Isl-1 and *Xlim-3* are expressed in certain subsets of neurons in the central nervous system as well as the pituitary and pineal glands, implying a similar function for LIM genes in vertebrate development (Thor *et al.*, 1991; Taira *et al.*, 1994a). While several LIM genes arise in cell type-specific patterns, *Xlim-1* is expressed at the gastrula stage in the dorsal blastopore lip and the dorsal mesoderm, a region known as the Spemann organizer. Since this region is responsible for establishing the dorsal axis, *Xlim-1* may play a global role in patterning rather than in cell type during gastrulation. Injection of *Xlim-1* constructs that contain point mutations in the LIM domains into *Xenopus* embryos can induce neural differentiation in animal explants and can induce secondary axis formation when

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ectopically expressed in the ventral side of whole embryos. A notable feature of the *Xlim-1* gene is that retinoic acid enhances its expression in animal explants (Taira *et al.*, 1992), as distinct from the response of other dorsally expressed genes such as *goosecoid* (*gsc;* Cho *et al.*, 1991) and *XFKH1*/*XFD-1*/*Pintallavis* (Dirksen and Jamrich, 1992; Knöchel *et al.*, 1992; Ruiz i Altaba and Jessell, 1992). Expression of *Xlim-1* in animal explants from *Xenopus* blastulae can also be induced by activin A and by retinoic acid. *Xlim-1* continues to be expressed from the early gastrula to the later stages in embryos in a biphasic manner: the level of expression peaks at late gastrula, then decreases, and increases again at the tail-bud stage. *Xlim-1* RNA is also expressed in several adult tissues including the brain, eye, kidney, and testis (Taira *et al.*, 1992). *Xlim-1* is expressed in three distinct cell lineages: those leading to notochord, pronephros, and certain subsets of cells in the central nervous system (CNS). The modification of the *Xlim-1* expression pattern by retinoic acid suggested a relationship between retinoic acid-induced malformations and enhanced *Xlim-1* expression (Taira *et al.*, 1992, 1994b).

Pronephros can be induced from presumptive ectoderm with activin A/retinoic acid treatment. *Xlim-1* expression is activated by activin A and by retinoic acid in animal explants, and cotreatment with activin A and retinoic acid leads to enhanced *Xlim-1* expression. Taken together, these results suggest that *Xlim-1*, the earliest known marker of pronephros, may function in pronephros organization. In this report, we examined the temporal expression of *Xlim-1* in animal caps with and without activin A/retinoic acid treatment. The patterns of *Xlim-1* expression in activin A/retinoic acid-treated animal explants suggested that *Xlim-1* is involved in pronephrogenesis. To investigate whether *Xlim-1* expression influences pronephros development, we injected the mRNA derived from *Xlim-1* clones with point mutations in the LIM domains (*Xlim-1*/3m) and from *Xlim-1* with the *Engrailed* repressor domain (*Xlim-1*-enR). The results of injection and induction experiments strongly suggested that *Xlim-1* is prerequisite for pronephros development.

MATERIALS AND METHODS

Eggs and embryos. Eggs of *Xenopus laevis* were obtained by the injection of human chorionic gonadotropin (Gestron; Denka Seiyaku, Japan). Both males and females were injected with 600 IU of gonadotropin. Staging of embryos was according to Nieuwkoop and Faber (1956). The jelly coat was removed with Steinberg's solution containing 4.5% cysteine hydrochloride (pH 7.8).

Microinjection. Eggs were fertilized *in vitro*, dejellied with 2% cysteine (pH 7.8), and incubated at 20°C in Steinberg's solution. Embryos were allowed to develop up to various stages before injection. *Xlim-1* constructs were kindly provided by I. Dawid (NIH, U.S.A.) and M. Taira (University of Tokyo, Japan). *Xldb-1*, *Xlim-1*, *Xlim-1*/3m, and β-galactosidase cDNAs had all previously been cloned into pSP64T. *Xlim-1*-enR had been cloned into pGEM/4Z. Capped mRNA was produced by *in vitro* transcription (mMessage mMachine kit; Ambion). For *Xlim-1*, *Xlim-1*/3m, *Xldb-1*, and β-galactosidase, 250 pg of mRNA was injected for single injections. In co-injection experiments with *Xlim-1* plus *Xldb-1*, *Xlim-1*/3m plus *Xlim-1*-enR, and *Xlim-1*-enR plus β-galactosidase, a total of 500 pg of mRNA was used for co-injections. In dose-dependent injection experiments of *Xlim-1*-enR, the dosage of mRNA was changed from 10 to 250 pg. These are described in detail in the results and figure legends. Embryos for injection were transferred to 5% Ficoll/Steinberg's solution and arranged on a grid. Embryos that were used for induction experiments were allowed to develop until stage 9 in Steinberg's solution containing 5% Ficoll.

Preparation of test solution. Human recombinant activin A (kindly provided by Dr. Yuzuru Eto, Central Research Laboratory, Ajinomoto Co., Kawasaki, Japan) was dissolved in Steinberg's solution containing 100 mg/L kanamycin sulfate and 0.1% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) at a concentration of 100 ng/ml and was stored at −80°C. Solutions were mixed to yield the following final concentrations: activin A 10 ng/ml, retinoic acid 10^{-4} mol/L, BSA 0.1% in Steinberg's solution.

Animal caps assay. The vitelline membrane of the blastula embryos was removed with fine forceps and presumptive ectoderm was isolated with tungsten needles. Both presumptive mesodermal and endodermal cells were removed from isolated ectoderm. Ectoderm sheets were immediately transferred to various test solutions and incubated for 3 h in a 24-well multititer plate (Sumilon MS-8024; Sumimoto Bakelite, Tokyo, Japan). After being washed with Steinberg's solution twice, explants were cultured at 20°C in Steinberg's solution. For histological examination, explants were fixed after a 4-day incubation (Fig. 4).

RT-PCR. For amplification of cDNAs encoding pronephric marker genes, total RNA was extracted from animal explants. Oligo(dT) primer was used to synthesize first-strand cDNAs from 1 μg of total RNA. Five primer sets described below were used for PCR: *Xlim-1* sense, TGGTGGACAGATTAGAGCCG, antisense, GTGATCCATTGCACCAAGG; *Xpax-2* sense, ATGGATATGCACTGCAAGGC, antisense, TCTTGCTCACACATCCATGG; *Xpax-8* sense, TATGCCTCCTCTGCAATTGC, antisense, TAAGGTCATAGGCTCCTGCC; *Xwnt-4* sense, GAGGTGATGGATTCAGTCCG, antisense, TGCACAGTCCTATCACAGCC; *ODC* sense, GTCAATGATGGAGTGTATGGATC, antisense, TCCATTCCGCTCTCCTGAGCAC.

One-tenth of the cDNA preparation was used as template for subsequent amplification by PCR. The amplification parameters of marker genes were *Xlim-1* and *Xpax-8*, 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 25 cycles followed by 7 min at 72°C; *Xpax-2*, 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s for 26 cycles; and *Xwnt-4*, 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s for 26 cycles. *ODC* primers were used at 20 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. One-tenth of the amplification products was resolved by 2% agarose gel electrophoresis.

In situ hybridization. Single-probe *in situ* hybridization was performed according to Harland (1991). Antisense digoxigenin-labeled RNA probe was transcribed from DNA from a full-length *Xldb-1*construct. For antisense mRNA transcription, *Xldb-1* was subcloned into pCS2+. DNA was linearized by *Eco*RI and transcribed with T7 RNA polymerase to generate antisense transcripts. A developmental series of albino embryos ranging in stage from midneurula to tadpole was hybridized with the labeled probes. The color reaction was performed using BM purple (Boehringer Mannheim Biochemica).

Immunohistochemistry and histological examination. Specimens were fixed in Bouin's solution for 3 h. They were then dehydrated through a graded series of ethanol, cleared in xylene, embedded in paraffin (Histprep 568; Wako, Japan), and sectioned at 6 μm. Sections were stained with Delafield's hematoxylin/eosin. For immunohistochemistry, specimens were fixed in MEMFA for 1 h at 4°C, assayed for β-galactosidase activity, refixed for 30 min, washed in PBS, and dehydrated in methanol overnight. Embryos were rehydrated in PBS, blocked with 20% sheep serum in PBS with 0.2% BSA and 0.1% Triton X-100, and then incubated in primary antibody such as pronephric tubule-specific antibody 3G8 or duct-specific antibody 4A6 at 4°C overnight. After five 1-h washes, sections were blocked and incubated in alkaline phosphatase-conjugated secondary antibody at 4°C overnight, washed again, and stained using NBT/TCIP. For double staining with 3G8 and 4A6, specimens were fixed in MEMFA, washed three times in PBS, and probed for the second antibody 4A6. In double staining, the red substrate of a multicolor detection kit was used for 4A6. After staining, specimens were fixed in MEMFA again and dehydrated in methanol overnight. Wild-type embryos and animal caps were bleached in 10% H_2O_2 for 6 h. Specimens were transferred to ethanol and xylene, then embedded in paraffin. Embedded specimens were sectioned at 10 μm.

RESULTS

Relevance of Pronephros Formation and Expression of Xlim-1 in Activin/Retinoic Acid-Treated Animal Caps

In *Xenopus* embryogenesis, *Xlim-1* is expressed in the organizer at gastrula stage and in pronephros primordium from stage 12.5 (Taira *et al.*, 1994b; Brennan *et al.*, 1999). Expression of *Xlim-1* is also reported to be activated by activin treatment, retinoic acid treatment, and activin/retinoic acid treatment in animal caps (Taira *et al.*, 1992). However, no pronephros development is seen in animal caps treated with retinoic acid or activin alone. To investigate the regulation and role of *Xlim-1* during pronephros development, we conducted the animal cap assay experiment to analyze the temporal expression pattern of *Xlim-1* in the animal caps that received various treatments.

Animal caps isolated from late *Xenopus* blastulae were incubated in Steinberg's solution for untreated control or with activin (10 ng/ml), retinoic acid (10^{-4} M), or activin (10 ng/ml) and retinoic acid (10^{-4} M), which is reported to induce the highest rate of pronephros induction for 3 h (Uochi and Asashima, 1996), washed twice, transferred to Steinberg's solution, and cultured for 0–24 h and then harvested for RT-PCR analysis. The expression of *Xlim-1* was observed under all treatment conditions, but not in the untreated control. In retinoic acid-treated animal caps, *Xlim-1* mRNA was not detected after the 15th hour from treatment. Expression of *Xlim-1* decreased from the 12th hour after activin treatment in animal caps. When reference embryos grew to the neurula stage, expression of *Xlim-1* declined in animal caps with treatments that induce no pronephros. In contrast to activin treatment, expression of *Xlim-1* was decreased temporarily at 12 h and rose again from 15 h after treatment in activin/retinoic acid-cotreated animal caps. The different expression patterns obtained with these treatments suggested that the expression of *Xlim-1* after 12 h was caused by a synergistic action of activin and retinoic acid and that expression of *Xlim-1* in this period could be related to pronephros development.

The temporal expression pattern of *Xlim-1* in activin/retinoic acid-treated animal caps indicated that *Xlim-1* could be temporarily activated by retinoic acid and by activin, but that both activin and retinoic acid were required for continuous expression of *Xlim-1*. Expression of *Xlim-1* is enhanced by retinoic acid in lateral mesoderm of the neurula (Taira *et al.*, 1994b), suggesting that sustained expression of *Xlim-1* induced by activin-retinoic acid cotreatment may be the result of retinoic acid regulation of activin-induced mesoderm in animal caps. If this hypothesis is true, changes in timing between activin treatment and retinoic acid treatment will affect pronephrogenesis in animal caps and the expression of pronephric genes including *Xlim-1*. To test this hypothesis, we conducted a serious of time-lag induction experiments (Fig. 1B). Animal caps isolated from late blastulae were treated with 10 ng/ml activin solution, transferred to Steinberg's solution, and cultured for 3–21 h and then treated with 10^{-4} M retinoic acid. After retinoic acid treatment, animal caps were transferred to Steinberg's solution and cultured to 4 days for histological examination or were harvested at 24 h after activin treatment to determine the expression of pronephric marker genes, including *Xlim-1* (Table 1 and Fig. 1C).

Table 1 and Fig. 1C show the results of an induction experiment. The incubation time between activin treatment and retinoic acid treatment affected the differentiation pattern in animal caps. Pronephros formation was observed at almost the same frequency as with activin/retinoic acid cotreatment, when retinoic acid treatment was performed within 9 h after activin treatment (Figs. 1C-1 and 1C-2). If the culture period between activin and retinoic acid treatment was extended to 12 h or more (Table 1, Fig. 1C-3), the rate of pronephros formation decreased, until finally no pronephros was observed in these animal caps (Fig. 1C-4). Moreover, no pronephros was detected in animal caps that were treated with retinoic acid before activin treatment (data not shown). Figure 1D shows the expression of pronephros markers in induction experiments. *Xpax-2* and *Xwnt-4* were expressed consistently and showed no marked differences with any treatment. Expression of *Xlim-1* was detected in animal caps treated with a 3- and 12-h time lag between activin and retinoic acid, but not at 15 h or later. *Xpax-8* also showed an expression pattern similar to that of *Xlim-1*. Compared with the results of Table 1, only expression of *Xlim-1* and *Xpax-8* decreased under the conditions in which the rate of formation of pronephros decreased in animal caps. These results suggested that *Xlim-1* could directly affect pronephrogenesis, since *Xlim-1* expression paralleled pronephros development under these various conditions. Furthermore, these results of the temporal expression and time lag experiment may

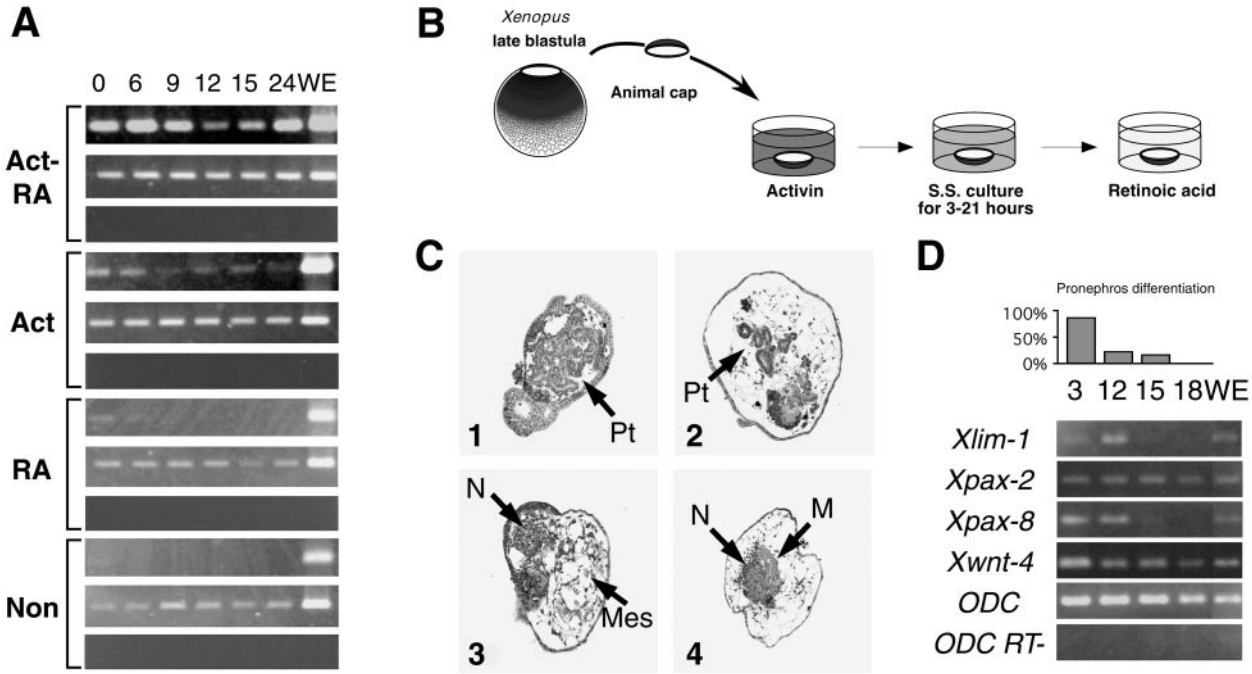

FIG. 1. Differentiation of pronephros in activin/retinoic acid-treated animal caps. (A) Temporal expression patterns of *Xlim-1* in animal caps. Animal caps were treated with 10 ng/ml activin and 10^{-4} M retinoic acid (Act-RA), 10 ng/ml activin (Act), or 10^{-4} M retinoic acid (RA) or were untreated (Non). Expression of *Xlim-1* (top in each treatment), *ODC* (middle) as a positive control, and sample without reverse transcriptase as a negative control (bottom) were included in all treatments. In activin/retinoic acid-treated animal caps, *Xlim-1* was expressed immediately after treatment, decreased at 12 h, and then increased again to be constantly expressed by 15 h. In activin A-treated animal caps, *Xlim-1* was also expressed immediately after treatment, but expression had decreased by 9 h. In retinoic acid-treated animal caps, *Xlim-1* was weakly expressed until 6 h. No *Xlim-1* mRNA was detected in untreated animal caps. (B) Time-lag induction with activin and retinoic acid. Animal caps isolated from late blastulae were treated with 10 ng/ml activin solution, transferred to Steinberg's solution, and cultured for 3–21 h and then treated with 10^{-4} M retinoic acid. After retinoic acid treatment, animal caps were transferred to Steinberg's solution and cultured to 4 days for histological examination or were harvested at 24 h after activin treatment to determine the expression of pronephric marker genes including *Xlim-1*. (C) Differentiation patterns of animal caps in the induction assay. (C-1 and C-2) Pronephros was detected in animal caps cultured for 3 (C-1) or 12 h (C-2) between activin A and retinoic acid treatment. (C-3) Frequency of pronephros differentiation markedly decreased in explants treated with a 15-h incubation between activin A and retinoic acid treatment. In this image, no pronephros differentiation is observed in the explant. (C-4) The synergistic action of activin A/retinoic acid disappeared when the incubation period was more than 18 h. No pronephros was observed in explants. Pt, pronephric tubule. N, neural tissue. Mes, mesenchyme. M, muscle. (D) Expression of pronephric marker genes in activin A/retinoic acid-treated animal caps. All animal caps were harvested at 24 h after activin A treatment. Numbers above the lanes show the time prior to retinoic acid treatment. Expression of *Xlim-1* and *Xpax-8* diminished when culture time was extended to 15 h. No obvious changes were detected in expression of *Xpax-2* or *Xwnt-4*.

reveal the activating mechanism of *Xlim-1* in activin/retinoic acid-treated animal caps.

Xlim-1 Is Required for Pronephros Development in Activin/Retinoic Acid-Treated Explants

The results of RT-PCR suggested the relevance of the pronephros differentiation in the animal cap with activin/retinoic acid treatment and expression of *Xlim-1*. To clarify the position of *Xlim-1* during pronephros development, we performed an injection experiment on *Xlim-1* mutants. As Fig. 2A illustrates, three *Xlim-1* constructs, *Xlim-1*, *Xlim-1*/3m, *Xlim-1*-enR, and *Xldb-1*, were used for these injection experiments (Fig. 2A). *Xldb-1* is an activating factor of *Xlim-1* which combines with its LIM domains and releases the DNA transcription domain of *Xlim-1* (Breen *et al.*, 1998). Expression of *Xldb-1* is observed in the CNS, pronephros, and tail bud in neurula and tail-bud stage embryos (Figs. 2B and 2C). *Xlim-1*/3m, which has a double point mutation in both LIM domains, retains activity (Taira *et al.*, 1994a). *Xlim-1*-enR is a fusion mutant with an *engrailed* repressor domain which should repress the function of *Xlim-1*. To verify whether these *Xlim-1* constructs can work *in vivo*, *Xlim-1*, *Xlim-1*/3m, *Xlim-1*-enR, and *Xldb-1* mRNA was synthesized *in vitro* and injected into ventral vegetal cells (*Xlim-1*/*Xldb-1* and *Xlim-1*/3m) and dorsal-vegetal cells (*Xlim-1*-enR) of eight-cell stage *Xenopus* embryos. Partial secondary axis formation was recognized in

TABLE 1
Animal Caps Cut Out from Late Blastulae for Various Culture Times between Activin A and Retinoic Acid Treatment

	Induction assay													
	3 h		6 h		9 h		12 h		15 h		18 h		21 h	
Neural tissue	0	0%	0	0%	10	59%	14	74%	14	70%	13	68%	16	64%
Pronephros	13	87%	17	57%	7	41%	4	21%	3	15%	0	0%	0	0%
Muscle	1	7%	7	23%	12	71%	17	89%	14	70%	12	63%	16	64%
Epidermis	14	93%	16	53%	17	100%	18	95%	20	100%	18	95%	24	96%
Mesenchyme	6	40%	3	10%	1	6%	15	79%	15	75%	13	68%	18	72%
Coelic epidermis	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Endodermal cell	0	0%	0	0%	0	0%	4	21%	4	20%	2	11%	3	12%
Notochord	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Cement gland	4	27%	1	3%	1	6%	0	0%	1	5%	1	5%	4	16%
Total	15		30		17		19		20		19		25	

Note. As shown, the frequency of pronephros organization decreased while culture time between activin A and retinoic acid treatment increased over 9 h.

Xlim-1/Xldb-1 co-injected embryos and *Xlim-1*/3m-injected embryos, as reported previously (Fig. 2D; Taira *et al.*, 1994a). In *Xlim-1*-enR-injected embryos, the head structure was truncated, and the anterior somites and pronephros failed to form (Fig. 2E).

Xlim-1 was reported to induce ectopic pronephros from intermediate mesoderm of *Xenopus*. We examined the effect of injection of *Xlim-1*/3m, *Xlim-1,* and *Xlim-1+Xldb-1* mRNA into *Xenopus* embryos on subsequent treatment of animal caps of these embryos isolated at late blastula stage and cultured in the presence of retinoic acid solution or activin, which normally specifically induce mesodermal tissue formation (Asashima *et al.*, 1990; Arizumi *et al.*, 1991). Table 2 shows the results of these injection experiments. Animal caps from *Xlim-1*-, *Xlim-1+Xldb-1*-, and *Xlim-1*/3m-injected embryos that were untreated or treated with retinoic acid (10^{-4} M) differentiated into cement gland and also showed slight neuralization (Fig. 3D). No pronephros was detected. Animal caps from *Xlim-1*-, *Xlim-1+Xldb-1*-, and *Xlim-1*/3m-injected embryos treated with activin (10 ng/ml) differentiated into neural tissues, muscle, notochord, and other tissues but no pronephros was observed (Table 2 and Fig. 3E). Since pronephros was not induced by overexpression of *Xlim-1* in the mesodermal environment induced by activin, *Xlim-1* may induce pronephrogenesis from a specialized mesodermal environment such as early pronephric primordium, but not from the common mesodermal tissues.

We then examined the effect of a functional deficit of *Xlim-1* on pronephrogenesis. Animal caps were injected with *Xlim-1*-enR and cultured in Steinberg's solution for 4 days after a 3-h activin and retinoic acid treatment, which normally specifically induces pronephros formation (Moriya *et al.*, 1993). Pronephrogenesis was dose-dependently inhibited by *Xlim-1*-enR. In animal caps injected with 250 ng/embryo of *Xlim-1*-enR, pronephros differentiation after activin and retinoic acid treatment of animal caps was markedly inhibited by 25 to 75% (Table 3). This result was confirmed by immunohistochemistry with pronephric tubule-specific antibody 3G8 (Figs. 3A–3C). In animal caps in which pronephros differentiation was inhibited by *Xlim-1*-enR, there was a noticeable increase in muscle and neural tissue differentiation (Fig. 3F). Co-injection of *Xlim-1*/3m and *Xlim-1*-enR increased the frequency of pronephros differentiation to 57% (Table 2; Fig. 3G), confirming that this effect was due to specific inhibition of *Xlim-1* by *Xlim-1*-enR. Co-injection of *Xlim-1*/3m and *Xlim-1*-enR also decreased the frequency of muscle differentiation. Figure 3H shows the effects of *Xlim-1* constructs on expression of other genes. In induction experiments, all the explants were harvested at 24 h after treatment. In *Xlim-1*/3m-injected explants, no expression of any other marker gene was induced with any treatment, including activin treatment. The results of RT-PCR corresponded with histological analyses and demonstrated that overexpression of *Xlim-1* never induced pronephros marker gene expression in explants.

A Functional Deficit of Xlim-1 Causes Failure of Tubulogenesis in Pronephros Development

A functional deficit of *Xlim-1* resulted in failure of pronephros development in animal caps. Although muscle differentiation was observed in *Xlim-1*-enR-injected animal caps, expression of *Xlim-1*-enR in the dorsal-vegetal site of embryos inhibited the development of anterior somites. As anterior somites are necessary for development of pronephros (Mauch *et al.*, 1999; Seufert *et al.*, 1999), development of pronephros might be inhibited indirectly by *Xlim-1*-enR via malformation of anterior somites. To verify this possibility, we injected *Xlim-1*-enR into the lateral marginal zone of embryos. *Xlim-1*-enR mRNA (250 pg) was injected

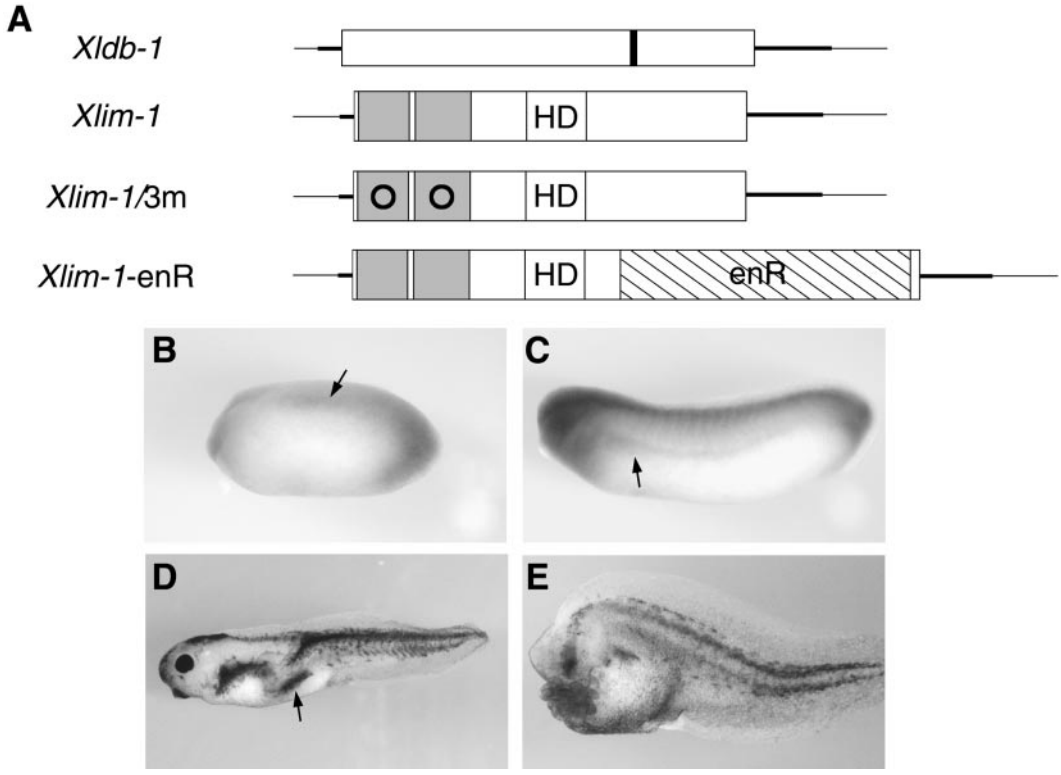

FIG. 2. (A) Constructs used for injection experiments. *Xldb-1* is an activator of *Xlim-1*. The solid box assigned to the C-terminal is the LIM-binding domain. *Xlim-1*/3m, the *Xlim-1* mutant with double point mutations in the LIM domains, is a constructively active form of *Xlim-1* (Taira *et al.,* 1994). The fusion mutant of *Xlim-1* with the repressor domain from *Engrailed* (*Xlim-1*-enR) can repress *Xlim-1* function. (B and C) Expression of *Xldb-1* in *Xenopus* embryos. *Xldb-1* is expressed in CNS, pronephros, and tail bud of *Xenopus* embryos. In stage 23 (B) and stage 28 (C) embryos, expression of *Xldb-1* is observed in the pronephros (arrows). (D and E) Effects of *Xlim-1* mutant. (D) Expression of *Xlim-1*/3m in the ventral side of embryos resulted in secondary axis formation as reported (Taira *et al.,* 1994). (E) Injection of *Xlim-1*-enR led to failure of the head structure to develop. A transverse section of this embryo indicated that development of anterior somites and pronephros was severely inhibited (not shown).

into the marginal zone of 32-cell stage *Xenopus* embryos along with 250 pg of mRNA encoding β-galactosidase as a lineage tracer. Both the C-2 and the C-3 blastomeres of embryos were injected with mRNA (Dale and Slack, 1987), and these blastomeres correspond to cells of the presumptive anterior somites (C-2), posterior somites, pronephros, and lateral plate (C-3). To investigate the functional effects of *Xlim-1*-enR, injected embryos were raised to stages 31/32 and 33/34 and then fixed with MEMFA. Pronephros development was examined by staining of pronephric tubules with the pronephric tubule-specific antibody 3G8. Only embryos that were phenotypically normal and had β-galactosidase activity as detected in the region of the pronephros were scored for effects on pronephric development (Table 4).

Figure 4 shows the results of this experiment. Normally, in stage 31/32 *Xenopus* embryos the pronephros condenses, pronephric tubules extend, and the entire structure starts to be lumenized (Vize *et al.,* 1995, 1997). In *Xlim-1*-enR-injected embryos, the area of 3G8 staining in the mesenchyme was almost the same as that of stage 32 control embryos (Fig. 4). Although we did not check for unlumenized structure, the main lumenized structures of the pronephros in *Xlim-1*-enR-injected embryos developed normally. In stage 33/34 embryos, the entire pronephric tubules were stained by 3G8. The pronephric tubules consist of three dorsal branches, or connective tubules, linked to a common tubule, which links to the pronephric duct (Fig. 4). At this stage, injection of *Xlim-1*-enR suppressed the development of pronephric tubules. In most *Xlim-1*-enR-injected embryos, development of the connective tubules was inhibited while the common tubule developed normally (Fig. 4). In severe case, dorsal branches of pronephric tubules were absent in the injected side. In control β-galactosidase-injected embryos, pronephric tubules developed normally (Fig. 4).

These results suggested that *Xlim-1*-enR inhibits the development of pronephric tubules. However, the common tubule of pronephric tubules was always present in *Xlim-1*-enR-injected embryos. *Xlim-1* is also expressed in the

TABLE 2
Results of *Xlim-1* and *Xlim-1*/3m Microinjection

	Xlim-1						*Xlim-1* and *Xldb-1*						*Xlim-1*/3m					
	Non		RA		Act		Non		RA		Act		Non		RA		Act	
Neural tissue	0	0%	0	0%	14	78%	2	11%	4	19%	19	83%	8	31%	7	23%	19	83%
Pronephros	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Muscle	0	0%	0	0%	8	44%	0	0%	0	0%	9	39%	0	0%	0	0%	11	48%
Epidermis	21	100%	18	90%	17	94%	18	100%	20	95%	22	96%	25	96%	28	93%	22	96%
Mesenchyme	0	0%	4	20%	0	0%	0	0%	3	14%	4	17%	0	0%	4	13%	0	0%
Coelic epidermis	4	19%	0	0%	0	0%	7	39%	0	0%	2	9%	4	15%	0	0%	0	0%
Endodermal cell	0	0%	2	10%	10	56%	0	0%	4	19%	7	30%	0	0%	15	50%	12	52%
Notochord	0	0%	0	0%	6	33%	0	0%	0	0%	5	22%	0	0%	0	0%	8	35%
Cement gland	2	10%	2	10%	1	6%	5	28%	0	0%	0	0%	5	19%	0	0%	2	9%
Total	21		20		18		18		21		23		26		30		23	

Note. In the *Xlim-1*/3m injection experiment, all embryos were injected with 250 pg of *Xlim-1* mRNA. In *Xlim-1* and *Xldb-1* co-injection experiments, all embryos were injected with 250 pg of *Xlim-1* and 250 pg of *Xldb-1* mRNA. Animal caps were removed at late blastula, treated with activin A or retinoic acid, and cultured for 4 days for histological analyses.

pronephric duct, which is linked to the common tubule from early neurula stage to tail-bud stage. In order to investigate if the pronephric duct was also inhibited by *Xlim-1*-enR, *Xlim-1*-enR plus β-galactosidase-co-injected embryos were raised to stage 40, when the duct can be detected by the 4A6 antibody, and fixed for immunohistochemistry. Embryos were double stained using both 3G8 and 4A6 to detect the changes in pronephric tubules and duct (Fig. 5; Table 4). As Fig. 5 shows, *Xlim-1*-enR did not inhibit the development of the pronephric duct, which was essentially normal in appearance. Although the length of the anterior coil in the duct was reduced, the structure of the pronephric duct, in particular the posterior portion, was normal. The diameter of the pronephric duct was also normal. In these injected embryos, the structure of the pronephric duct including the junction to tubules developed normally. The pronephric tubules showed the same phenotype as that of stage 33/34 embryos with *Xlim-1*-enR injected. These results suggested that *Xlim-1*-enR inhibited the growth of pronephric tubules, especially the growth of dorsal branches from lumenization of pronephric tubules.

Injection of *Xlim-1*-enR into the dorsal-vegetal blastomeres of 8-cell embryos led to failure of development of anterior somites and pronephros. Injection into the lateral marginal zone of 32-cell stage embryos also resulted in inhibition of anterior somite development. As anterior somites are necessary for the differentiation of pronephros, it was important to determine whether the failure of pronephric tubule formation was due to a secondary effect of *Xlim-1*-enR on the development of other structures such as the somites. Stage 40 embryos from the injection experiments were examined by gross examination and histology. A defect of anterior somites was detected by histology and appearance because a single side defect in anterior somites causes bending of the embryo axis. We quantitated the effects of *Xlim-1*-enR on pronephros in each embryo (Table

FIG. 3. Induction experiments in *Xlim-1*-mutant-injected animal caps. Two-cell stage embryos were injected with 250 pg/embryo *Xlim-1* mutant (*Xlim-1*/3m, *Xlim-1*-enR). (A–C) Immunohistochemistry of activin/retinoic acid-treated animal caps. All animal caps were cotreated with activin and retinoic acid for 3 h. *Xlim-1*-enR inhibited pronephros differentiation in a dose-dependent manner. Animal caps were bleached in H_2O_2 for 6 h after staining. (A) Differentiation of the pronephros was detected by the pronephric tubule-specific 3G8 antibody in control activin A/retinoic acid-treated animal caps. Arrows indicate signals (dark blue) of 3G8. In animal caps injected with 50 pg of *Xlim-1*-enR (B) and animal caps injected with 250 pg of *Xlim-1*-enR (C) the frequency of pronephros differentiation (41% in this experiment) was markedly decreased compared to animal caps injected with 50 pg of *Xlim-1*-enR (72%). (D–G) Differentiation patterns in *Xlim-1* construct-injected animal caps. (D) *Xlim-1*/3m-injected untreated animal cap. Cement gland was observed (arrow). (E) *Xlim-1*/3m-injected animal cap with activin treatment. Arrows indicate notochord (Nc) and neural (N) tissues in animal cap. (F) Animal cap (injected with *Xlim-1*-enR) treated with activin A and retinoic acid. Though muscle and mesenchyme tissues were recognized (arrows), no pronephros was detected. (G) Pronephros (arrows) was recognized in *Xlim-1*/3m and *Xlim-1*-enR co-injected animal caps treated with activin A and retinoic acid. Cg, cement gland. N, neural tissue. Nc, notochord. M, muscle. Mes, mesenchyme. Pt, pronephric tubule. (H) Overexpression of *Xlim-1* induced no pronephros marker genes. Lanes 1–3: *Xlim-1*/3m-injected explants. There is no expression of pronephric marker genes detected in *Xlim-1*/3m-injected explants with activin A (lane 1) or retinoic acid (lane 2) or in controls (lane 3). Lane 4: activin A/retinoic acid cotreated explants. Lane 5: sample from the whole embryo.

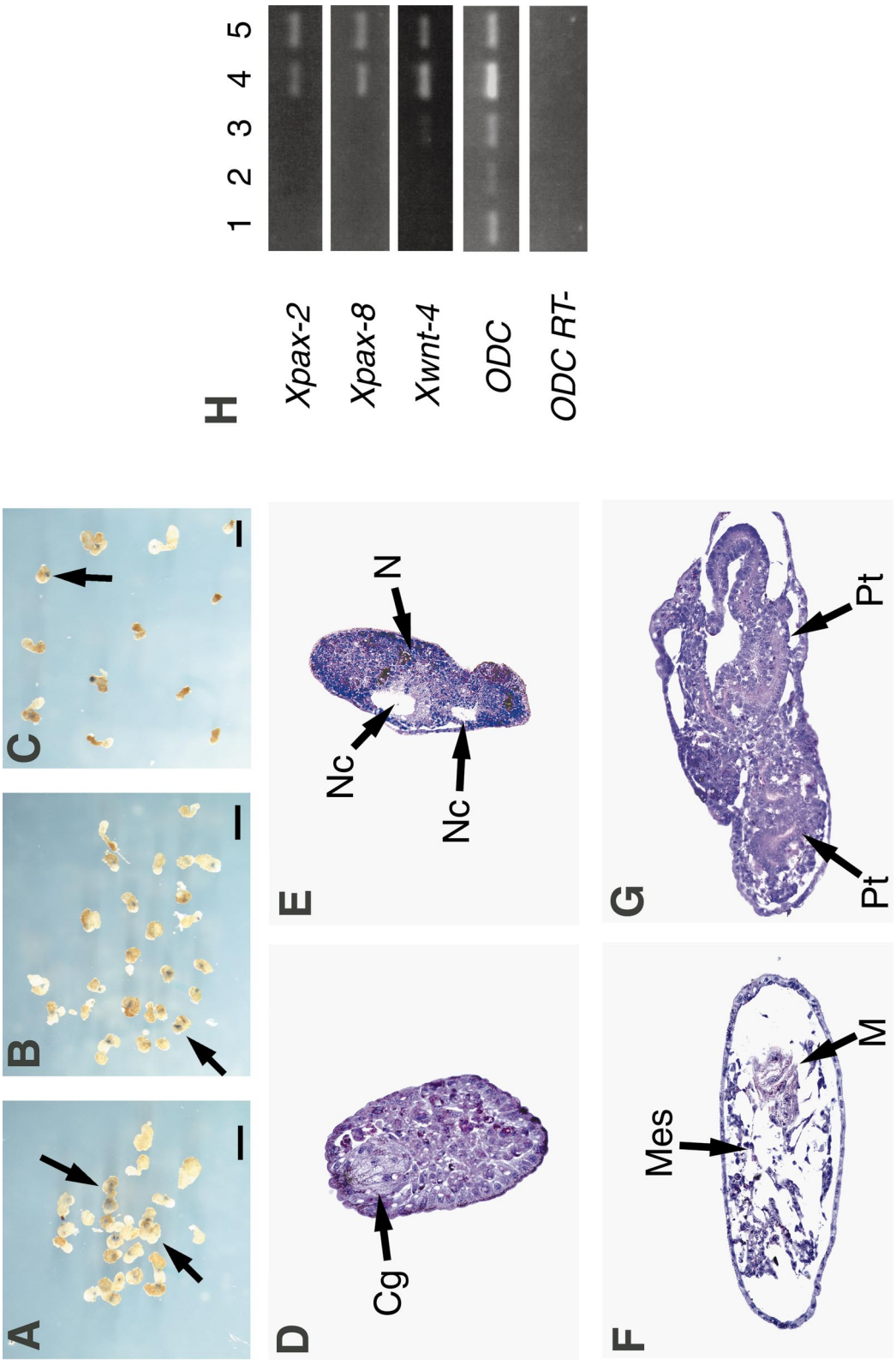

TABLE 3
Results of *Xlim-1*-enR Microinjection

	Xlim-1-enR										*Xlim-1*/3m and *Xlim-1*-enR	
	Un-injected		10 pg		50 pg		100 pg		250 pg		Co-injected	
Neural tissue	2	10%	3	17%	6	38%	8	47%	15	47%	14	50%
Pronephros	15	75%	13	72%	8	50%	6	35%	8	25%	16	57%
Muscle	2	10%	2	11%	5	31%	7	41%	11	34%	7	25%
Epidermis	17	85%	15	83%	14	88%	16	94%	28	88%	25	89%
Mesenchyme	5	25%	6	33%	4	25%	4	24%	18	56%	10	36%
Coelic epidermis	4	20%	5	28%	4	25%	4	24%	9	28%	6	21%
Endodermal cell	0	0%	0	0%	2	13%	3	18%	10	31%	0	0%
Notochord	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Cement gland	0	0%	0	0%	0	0%	0	0%	0	0%	0	0%
Total	20		18		16		17		32		28	

Note. In *Xlim-1*-enR injection and co-injection (*Xlim-1*/3m and *Xlim-1*-enR) experiments, all explants were treated with activin A and retinoic acid. Animal caps were removed at late blastula, treated with activin A or retinoic acid, and cultured for 4 days for histological analyses.

4). A total of 42% of embryos had defects in anterior somites and had malformations in pronephros, including the duct, as reported (Mauch *et al.*, 1999; Seufert *et al.*, 1999). Severe failure of pronephros development was observed in the injected side of these embryos accompanied by a bent axis, but the pronephros of the opposite side developed normally (not shown). As we described above, injection of *Xlim-1*-enR into dorsal-vegetal cells of 8-cell stage embryos resulted in truncation of head structure and failure of anterior somite development. The C2 blastomere is a dorsal-lateral cell of 32-cell stage embryos; these effects of *Xlim-1*-enR on anterior somites are expected. On the other hand, about 58% of *Xlim-1*-enR-injected embryos developed with a normal axis and anterior somites. In embryos without any defect in anterior somites, inhibition of pronephric tubule formation was detected in 63% of injected embryos. The pronephric duct was normal in all of these embryos. Immunohistochemistry and histological analyses of these embryos showed that tubulogenesis was suppressed despite normal anterior somite development (Fig. 5). Somites underlying the malformed tubules formed normally without any obvious changes in size. These results suggest that *Xlim-1*-enR can inhibit development of pronephric tubules directly. As injection of *Xlim-1*-enR resulted in muscle differentiation in activin and retinoic acid-treated animal caps, enlargement of somites was expected in *Xlim-1*-enR-injected embryos. However, expression of *Xlim-1*-enR did not lead to enlargement of anterior somites despite disruption of tubule formation. This result may be because the reduced tubules in *Xlim-1*-enR-injected embryos were transferred into the mesenchyme around the pronephros (Fig. 5E).

TABLE 4
Injection of *Xlim-1*-enR

	Defects in somites		Normal appearance	
	st 40	st 33/34	st 40	st 33/34
++	11	14	4	4
+	12	14	25	17
−	0	7	17	12
Total	23	35	46	33

Note. Effects of *Xlim-1*-enR on pronephric tubules with/without failure of anterior somites. ++, the branches of pronephric tubules were absent or inhibited severely. Reduction in size of the common tubule was also counted for this defect level. +, one of the branches was inhibited, or the length of tubules was reduced in all three branches with normal common tubule.

DISCUSSION

The phenotype of the *lim-1* knockout mouse showed that *lim-1* is required for normal kidney formation (Fujii *et al.*, 1994). Co-injection of *Xlim-1* and *Xpax-8* also leads to ectopic development of pronephric tubules (Carroll and Vize, 1999). In this report, we have shown that the development of pronephric tubules, but not pronephric duct, was suppressed by injection of *Xlim-1*-enR, a functional deficit mutant of *Xlim-1*. Moreover, overexpression of *Xlim-1* never induced pronephros differentiation in animal caps treated with activin. This suggests that the role of *Xlim-1* in pronephrogenesis is mainly in tubulogenesis, instead of formation of pronephric primordium.

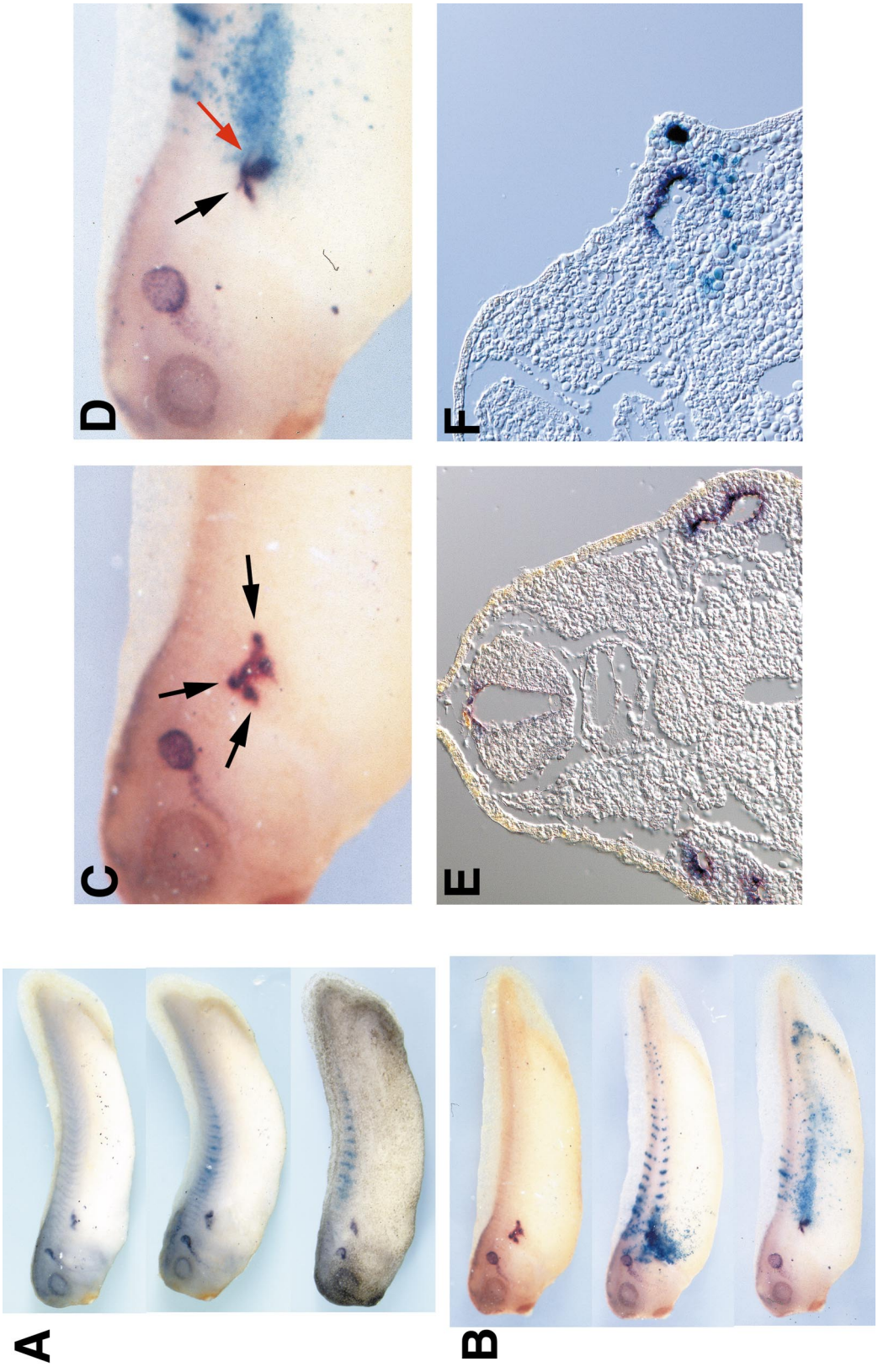

FIG. 4. Effects of *Xlim-1*-enR on pronephros development. (A and B) *Xlim-1*-enR-injected embryos. *Xlim-1*-enR and β-galactosidase were injected into C2 and C3 blastomeres (left side) of 32-cell stage embryos. Embryos were fixed at stage 31/32 (A) and stage 33/34 (B), and then development of pronephric tubules was analyzed by staining with 3G8. (A) Pronephric tubules of st 31/32. The embryo injected with *Xlim-1*-enR is on the bottom. An embryo injected with β-galactosidase alone was used for injection control (middle). Pronephric tubules in *Xlim-1*-enR-injected embryo have the same appearance as the control embryo (top in A). (B) Pronephric tubules of stage 33/34 embryos. Arrangement of embryos is the same as in A. The effects of *Xlim-1*-enR during pronephros development are detectable at this stage. In the *Xlim-1*-enR-injected embryo (bottom), reduced pronephric tubule development was observed. Details are described in D–F. (D) *Xlim-1*-enR inhibited tubulogenesis. Arrows indicate reduced development of the dorsal branches of pronephric tubules. Compared to C, the most anterior branch of the connective tubule is slightly reduced. The length and diameter of another anterior dorsal branch is severely reduced (black arrow), and the posterior dorsal branch of tubules (red arrow) is almost absent. (E and F) Histological analysis of reduced pronephric tubules. Dorsal is up and lateral is right. (E) Control embryo. Transverse section through a normal pronephros, pronephric tubules are stained with 3G8 in dark blue. (F) Transverse section of *Xlim-1*-enR shown in D. The structure of the reduced tubules shows that the common tubule is lumenized normally. These results suggest that *Xlim-1*-enR suppresses the growth of tubules but not lumenization during tubulogenesis.

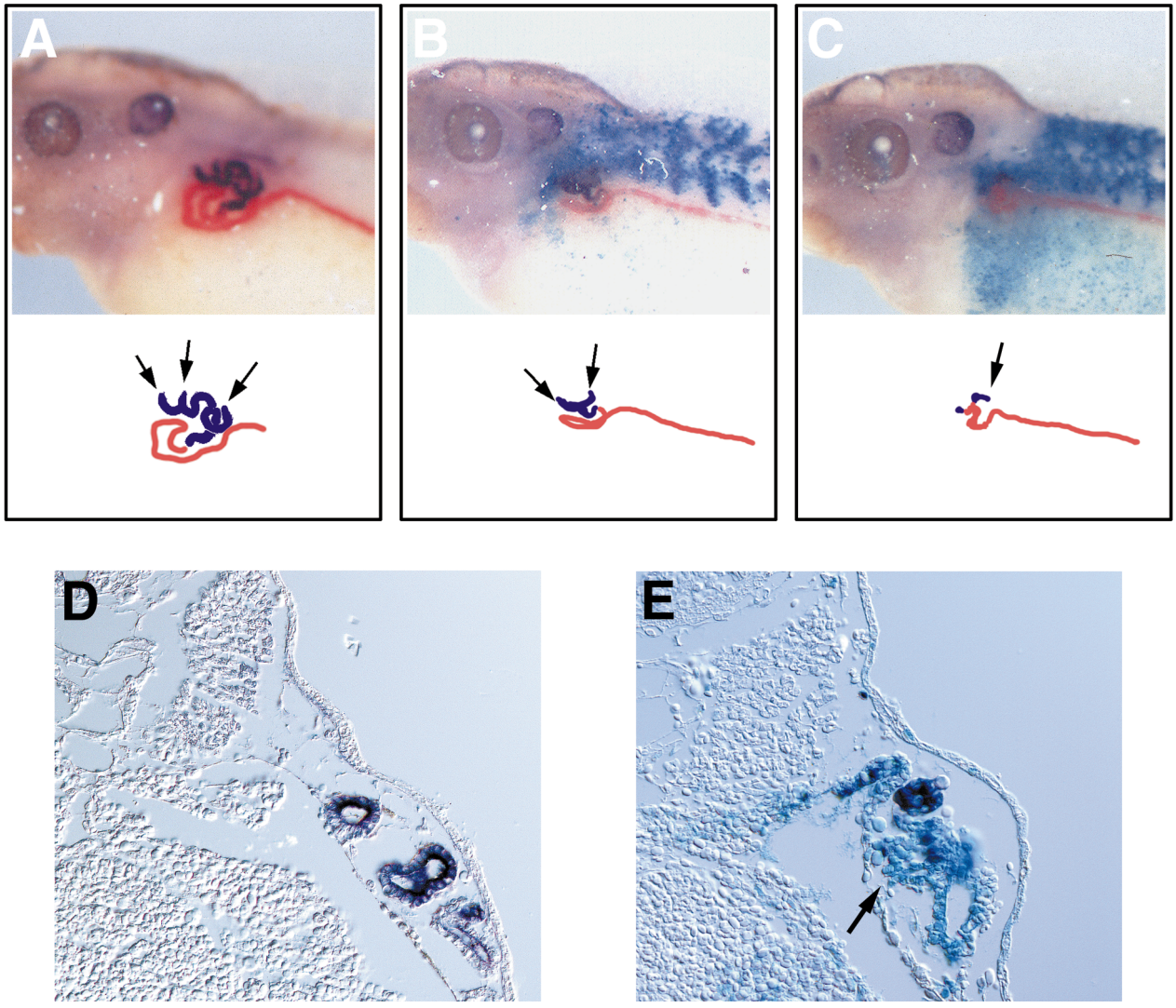

FIG. 5. Injection of *Xlim-1*-enR did not inhibit development of pronephric duct. All embryos were stained with antibodies 3G8 (dark blue) and 4A6 (red). (A) Normal pronephros of a stage 40 embryo. In the illustration below, arrows indicate the three normal dorsal branches. (B and C) Inhibited pronephros in *Xlim-1*-enR-injected embryos. These embryos showed a normal phenotype before staining. (B) Faint staining of the posterior dorsal branch of the pronephric tubules is observed. The length of the dorsal branches is reduced, and only one anterior dorsal branch is detected (arrows). The common tubule is also shorter than normal pronephric tubules. (C) Severe inhibition of development of an *Xlim-1*-enR-injected embryo. Arrow indicates the reduced common tubule with a small posterior dorsal tubule. Length of S-shaped coil in pronephric duct is also reduced, but the diameter of the duct is normal (shown in E). (D and E) Transverse sections of embryos in A and C (D, normal embryo; E, *Xlim-1*-enR-injected embryo). Compared to normal pronephros in D, the pronephric tubule is severely reduced in size in *Xlim-1*-enR-injected embryos, but the structure of the pronephric duct is normal. As the appearance of these embryos suggests, somites are normal in both normal and *Xlim-1*-enR-injected embryos (arrow). This suggests that *Xlim-1*-enR can directly inhibit pronephric tubules without affecting anterior somites.

Regulation of Xlim-1 in Activin/Retinoic Acid-Treated Animal Caps

Pronephros differentiation can be induced by activin/retinoic acid treatment (Moriya *et al.*, 1993; Uochi and Asashima, 1996), with accompanying expression of pronephric marker genes, including *Xlim-1*, *Xpax-8*, and *Xwt1*, as reported (Taira *et al.*, 1992; Heller and Brandli, 1999; Brennan *et al.*, 1999). Our presented results indicate that the difference between the pattern of *Xlim-1* expression with activin treatment and that with activin/retinoic acid treatment, in which *Xlim-1* is expressed by 12 h after treatment, is likely to be related to pronephros develop-

ment. *Xlim-1* was reported to be expressed in the pronephros from stage 12.5 (Taira *et al.,* 1994b). Results of time-lag induction experiments indicated that retinoic acid could induce pronephros within a 12-h window after activin treatment. Taking these results together, the following mechanism of *Xlim-1* activation by activin/retinoic acid treatment will be proposed. In the animal cap treated with activin and retinoic acid, some unknown factor activated by retinoic acid enhances the expression of *Xlim-1,* which was initially activated by activin, and then a synergistic effect of activin and retinoic acid maintains *Xlim-1* expression. Expression of *Xlim-1* is directly activated by activin and indirectly activated by retinoic acid (Taira *et al.,* 1992). This suggests that at least two transcription mechanisms are involved in *Xlim-1* activation. The main mechanism of activation is mediated by activin and can induce temporary *Xlim-1* expression, which will decrease from 12 h after activin treatment. The second mechanism is activated indirectly by retinoic acid. This retinoic acid-activated mechanism can also induce *Xlim-1* expression independently, but only at low levels for a short period. However, these two transcription mechanisms can cooperatively induce sustained *Xlim-1* expression. It is still unknown whether retinoic acid participates in pronephros development in the normal embryo. In studies of the genomic structure of *lim-1,* HNF3β and pbx1 binding sites have been identified (Li *et al.,* 1999). It is possible that a similar mechanism also exists in *Xlim-1.* In *Xenopus* embryogenesis, the overall concentrations of retinoic acid are much lower than 10^{-4} M (Blumberg *et al.,* 1997), but local concentrations may be higher. Furthermore, expression of *Xlim-1* was enhanced in embryos treated with retinoic acid (Taira *et al.,* 1994b). Therefore signals from retinoic acid are likely to regulate the expression of *Xlim-1* and pronephrogenesis in *Xenopus* embryos.

Effect of Xlim-1 Mutants on Pronephros Development

Overexpression of *Xlim-1* and *Xlim-1*/3m induces ectopic pronephric tubules in intermediate mesoderm (Carroll and Vize, 1999). Since ectopic pronephros was detected only in intermediate mesoderm, we investigated whether *Xlim-1* can induce pronephros formation in a mesodermal environment such as activin-treated animal caps. No pronephros formation was observed in animal caps with overexpression of *Xlim-1,* even in those treated by activin. Since differentiation of mesodermal tissue and expression of pronephric marker genes, such as *Xpax-8,* are detected in animal caps with activin (Taira *et al.,* 1992; Heller and Brandli, 1999; Brennan *et al.,* 1999), *Xlim-1* is unlikely to initiate pronephros development in cooperation with these factors and is more likely to be involved in the specialization of pronephric tissues than establishment of pronephric primordium.

Xlim-1/3m was used as the active form of *Xlim-1* in this experiment. Injection experiments of *Xlim-1*/3m had almost the same effect as *Xlim-1* + *Xldb-1.* Although it was reported that the activity of *Xlim-1*/3m was weaker than that of *Xlim-1* + *Xldb-1* (Agulnick *et al.,* 1996), similar rates of differentiation were seen in the animal cap assay. This suggests that mutation in the LIM domains does not change the property of *Xlim-1.* In other reports, *Xlim-1*/3m is less effective at inducing ectopic pronephros (Carroll and Vize, 1999). It is likely that DNA transcription activity of *Xlim-1* is enhanced by combination with *Xldb-1.* As *Xlim-1* is expressed in the organizer of early gastrula and overexpression of *Xlim-1* in the ventral side leads to secondary axis formation, *Xlim-1* is considered to be a dorsalizing factor. *Xlim-1*-enR, the repressor-fusion mutant of *Xlim-1,* is therefore considered a ventralizing factor by repressing the function of *Xlim-1.* However, our data show that while *Xlim-1*-enR is a repressor of *Xlim-1,* it does not have a ventralizing action. First, expression of *Xlim-1*-enR in dorsal-vegetal cells of *Xenopus* embryos did not result in ventralization. These embryos had truncation of head structure and were defective in anterior somites, but ventral tissues developed normally. Moreover, differentiation of neural tissue and muscles instead of endodermal tissue was detected in activin/retinoic acid-treated animal caps expressing *Xlim-1*-enR. Therefore, the action of *Xlim-1*-enR is not mediated via ventralization or prevention of dorsalization, but by repression of *Xlim-1* target genes. The results of knockout experiments of *lim-1* also support this reasoning.

We used *Xlim-1*-enR as a repressor of *Xlim-1* to investigate the role of *Xlim-1* in pronephrogenesis. The effects of *Xlim-1*-enR on pronephros development were observed at stage 33/34, over 30 h after mRNA injection. We found that exogenous *Xlim-1*-enR mRNA persisted in the embryo for at least 24 h after injection (data not shown), but it is unlikely to persist to stage 40. Nevertheless, our data indicated that the injection of *Xlim-1*-enR inhibited tubulogenesis, which is a late event of pronephros development. One possible explanation for this is that early expression of *Xlim-1* may determine the position of pronephric tubules, and therefore *Xlim-1* expression is needed much earlier than the onset of tubulogenesis. During *Xenopus* development, *Xlim-1* is expressed from stage 12.5 in the pronephric primordium, and expression is sustained at the tip of pronephric tubules and duct to stage 36 (Taira *et al.,* 1994b; Carroll *et al.,* 1999). Expression of *Xlim-1*-enR in the lateral domain of *Xenopus* embryos may have suppressed the determination of pronephric tubules via blocking the target gene expression of *Xlim-1,* leading to failure of tubulogenesis.

The Role of Xlim-1 in Pronephrogenesis

Xlim-1, which is first expressed in pronephros at stage 12.5 and continues to be expressed until stage 36, is an important regulator of pronephros development. Base on the present results, we speculate that the role of *Xlim-1* in pronephros development is as follows. In early pronephrogenesis, the presumptive pronephric tubules are determined by expression of *Xlim-1.* Consistent expression of *Xlim-1*

mediates specialization and growth of pronephric tubules in pronephric primordium and is necessary until the tubules are lumenized. The expression pattern of *Xlim-1* also supports this speculation (Carroll *et al.,* 1999). *Xlim-1* is expressed in the whole pronephric primordium at neurula and early tail-bud stage, but only in the tip of pronephric tubules and duct at stage 36. In other reports, *Xwt-1* inhibited differentiation of pronephric tubules and suppressed the expression of *Xlim-1* (Wallingford *et al.,* 1998). Expression of *Xwt1,* the glomus marker. gene may mediate the development of glomus by suppressing expression of genes in pronephric tubules such as *Xlim-1.* Our data did not suggest any role for *Xlim-1* in development of the pronephric duct. Expression of *Xlim-1*-enR in the lateral portion of *Xenopus* embryos had no effect on development of the pronephric duct, while *Xlim-1* was also expressed in the pronephric duct (Carroll *et al.,* 1999). Differentiation of the pronephric duct is probably mediated by several genes including *Xlim-1,* and development of the pronephric duct may be rescued by other pronephric genes even if the function of *Xlim-1* is suppressed.

The results of the time-lag induction experiment with activin and retinoic acid suggested that *Xlim-1* and *Xpax-8* are directly related to pronephros development. *Xpax-8* expression is different from that of *Xlim-1;* it is found throughout the whole pronephric tubules of tail-bud stage embryos. Moreover, coexpression of *Xlim-1* and *Xpax-8* was reported to induce ectopic formation of pronephric tubules. Therefore *Xpax-8* may participate in specialization of pronephric tubules in cooperation with *Xlim-1,* perhaps via changing the transcriptional activity of *Xlim-1.* One example, the paired-class homeobox gene *Otx-2,* can bind to the homeodomain of *Xlim-1* directly and enhance transcription activity (Mochizuki *et al.,* 2000). It is possible that a similar interaction exists between *Xlim-1* and *Xpax-8.* Preliminary results have shown that co-injection of *Xlim-1* and *Xpax-8* downregulates the expression of some genes activated by *Xlim-1* in animal caps (data not shown).

Although injection of *Xlim-1* was reported to induce ectopic formation of pronephros in intermediate mesoderm of *Xenopus* embryos (Carroll and Vize, 1999), our data showed that injection of *Xlim-1* never induced pronephros in animal caps with activin treatment. Injection of *Xlim-1*-enR resulted in inhibition of pronephric tubule formation in embryos leading to an increase in muscle differentiation instead of pronephros differentiation in activin/retinoic acid-treated animal caps. *Xlim-1* determines differentiation of pronephric tubules but cannot induce pronephros independently, suggesting that other pronephric factors, which are expressed earlier than *Xlim-1* or *Xpax-8,* are involved in formation of pronephric primordium. It is already known that determination of pronephric tubules occurs at stage 12.5 and the determination of the pronephric duct occurs at stage 14 (Brennan *et al.,* 1998). Expression of *Xlim-1* in pronephros is also first detected at stage 12.5. Establishment of pronephric primordium may occur at an earlier stage than stage 12.5, and the differentiation of the pronephric tubules and duct is determined by expression of pronephric markers such as *Xlim-1* and *Xpax-8,* and then the boundary of the glomus and tubules is further determined by expression of *Xwt-1.* Muscle differentiation is observed in animal caps in which differentiation of pronephros was prevented such as in the *Xlim-1*-enR-injected animal caps treated with activin and retinoic acid and in the animal caps with time-lag treatment, in which expression of *Xlim-1* and *Xpax-8* was suppressed. Different from the intermediate mesoderm of embryos, there may be no distinct spatial arrangement of pronephros gene expression in activin/retinoic acid-treated animal caps. Therefore mesodermal tissues differentiate into muscle instead of other pronephric structures in *Xlim-1*-enR-injected animal caps. In summary, our data suggest that *Xlim-1* is essential for differentiation of pronephric tubules but not establishment of pronephric primordium. This also suggests that the determination of pronephric primordium is likely earlier than *Xlim-1* expression. More experimental data are still necessary for these hypotheses. Further analyses about the relation between the already-known pronephros marker genes and unknown factors will become an important step for elucidating the whole aspect of pronephros development.

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